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Mannose-binding lectin (MBL) genotype in relation to risk of nosocomial infection in pre-term neonates in the neonatal intensive care unit

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ABSTRACT

Mannose-binding lectin (MBL) plays an important role in the innate immune response. Three alleles in the *MBL* gene, and one allele of the promoter, independently cause low serum MBL levels as compared with the wild-type. This study investigated the relationship between MBL genotype and the occurrence of nosocomial infection among neonates in a neonatal intensive care unit (NICU). Prospectively gathered information concerning nosocomial infection was available for 742 neonates from a recently performed surveillance study in an NICU. DNA was isolated from Guthriecards for a subgroup of 204 neonates who stayed in the NICU for ≥4 days. After a pre-PCR for the *MBL* gene in blood spots on Guthriecards, mutations were analysed by real-time PCR to detect six mutations in the *MBL* gene. An MBL genotype could be determined for 186 neonates. As compared to term neonates, genotypes encoding MBL-deficient haplotypes were significantly more prevalent among pre-term neonates. Forty-one of these neonates developed sepsis, with blood cultures yielding coagulase-negative staphylococci in 25 cases. Pneumonia occurred in 30 cases, with various causative organisms. No relationship was found between MBL genotype and the risk of nosocomial sepsis or pneumonia, even after correction for birth-weight, perhaps because of an insufficient correlation between genotype and the concentration of functional MBL. In addition, most bloodstream infections in the NICU were caused by coagulase-negative staphylococci, to which MBL binds poorly.

Keywords Coagulase-negative staphylococci, haplotypes, immune response, mannose-binding lectin, neonates, nosocomial infection

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INTRODUCTION

The immune response consists of two separate systems. The first system comprises the aspecific innate immune system, which includes the complement system and mannose-binding lectin (MBL). The second system is the more specific adaptive immune system, which includes the range of white blood cells. The latter system is more effective, but is time-delayed. If the adaptive immune system is not compromised, the innate immune system is of minor importance [1]. However, if the adaptive immune system is compromised, the influence of any MBL deficiency becomes apparent [2,3].

MBL is a Ca^{2+}-dependent collagenous lectin, and has a bouquet-like structure that recognises and can bind to repeating pattern structures of polysaccharides on the surfaces of many micro-organisms. MBL functions as an ante-antibody, which means that it opsonises microorganisms before specific IgM can be produced [4]. Subsequently, an MBL-associated serine protease (MASP-2), which is functionally analogous to C1-esterase, activates the complement cascade that leads to killing of the microorganisms [5].

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The MBL monomer is encoded by the *MBL-2* gene on human chromosome 10, and functional MBL consists of a polymer of several MBL monomers. Three single base-pair substitutions in exon-1 of the *MBL-2* gene give rise to three mutant alleles (B, C and D), leading to failure of assembly of the functional MBL multimer. These polymorphisms occur in about one-third of the human population, with the frequency of occurrence of different polymorphisms being related to various ethnic groups [6]. The heterozygous polymorphisms reduce the amount of functional MBL by five- to ten-fold, whereas the homozygous mutations lead to almost undetectable titres of MBL [5]. Furthermore, several base-pair substitutions in the promoter region of the *MBL-2* gene have been described, leading to several promoter variants, some of which decrease the functional MBL serum level significantly [6].

The incidence of nosocomial infection in neonatal intensive care units (NICUs) is high [7]. The most important risk-factor in this patient category is low birth-weight [8,9], which is a risk-factor that, in part, reflects the more invasive treatment, e.g., with intravascular catheters and mechanical ventilation, of these infants. Furthermore, the adaptive host response to infection in neonates, especially pre-term neonates, is impaired for several reasons [10–15]. MBL deficiency may therefore be a risk-factor for infection in this patient category. In addition, an association between polymorphisms in the *MBL-2* gene and pre-term birth has been reported [16]. Accordingly, the present study investigated the influence of MBL genotype on the risk of nosocomial infection by determining the MBL-2 genotype of 204 neonates.

MATERIALS AND METHODS

Surveillance study

During 1998–2000, a prospective study was performed to investigate nosocomial bloodstream infection and pneumonia in 742 neonates admitted to the Neonatal Intensive Care Unit of the VU University Medical Centre. Local infection definitions were used, based on clinical and microbiological data [17], that were adapted from CDC infection definitions for children aged <1 year [18]. Several potential baseline risk-factors for nosocomial bloodstream infection and pneumonia were recorded. Neonates who stayed for >4 days in the NICU were eligible for inclusion in the study of MBL genotypes. The present study investigated retrospectively whether the MBL genotype could have been a risk-factor for nosocomial infection in the original surveillance study.

Investigation of single nucleotide polymorphisms (SNPs) in neonatal DNA

In The Netherlands, a blood sample is collected by heel puncture on to a Guthriecard from neonates during the first week after birth. These samples are investigated at the National Institute of Public Health and the Environment (Bilthoven, The Netherlands) for certain congenital diseases, and are then stored for 5 years. Parents of eligible neonates were asked for permission (informed consent) to use the Guthriecard of their child for DNA isolation and *MBL* gene analysis [19]. The study was approved by the local Ethical Committee.

Isolation of DNA. In brief, blood was eluted from Guthriecards by soaking three disks from each card in Tris-EDTA buffer (10 mM Tris-HCl, pH 8.3, 1 mM EDTA) and then incubating for 10 min at 56°C. The samples were then centrifuged at 16 100 *g* for 10 min. DNA was isolated from the resulting supernatant using a QIAamp kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and was eluted in 100 μL of the elution buffer contained in the kit. DNA samples were frozen at −20°C until use.

PCR. A nested PCR approach was used because the amount of DNA obtained from the Guthriecards was too small for direct detection of SNPs. A pre-PCR with two primers flanking the *MBL-2* gene was performed to amplify a DNA segment containing all six SNP targets. SNP analysis was then performed using six different real-time *Taq*Man assays [19]. Each assay consisted of non-labelled forward and reverse primers in combination with two fluorescent *Taq*Man oligonucleotide probes, labelled differently for wild-type and mutations. Because of the close proximity of the mutations in exon-1, these were enclosed by one primer pair. Oligonucleotides corresponding to wild-type promoter variants L, Y and P, and the wild-type structural gene A, were labelled with the fluorescent VIC molecule. Oligonuclotides corresponding to mutation promoter variants H, X, Q and the mutation haplotypes of the structural gene B, C and D were labelled with 6-carboxy-fluorescein (FAM). Endpoint detection was performed on an ABI Prism 7000 (Applied Biosystems, Warrington, UK), and the results were analysed using sequence detection software v.1.01 (Applied Biosystems).

In theory, there are $2^6 = 64$ possible combinations for six SNPs, but because of a strong linkage dis-equilibrium between promotor and exon, there are only seven well-known haplotypes: four promoter variants with a wild-type structural gene (HYPA, LYPA, LYQA and LXPA) and three haplotypes with a mutant structural gene (LYPB, HYPD and LYQC) [20]. Every individual expresses two MBL haplotypes.

Analysis of the effect of MBL genotype on risk of nosocomial infection

Genotypes were stratified in six combined promotor–exon groups (YA/YA, YA/XA, XA/XA, YA/O, XA/O and O/O) and in three exon groups (A/A, A/O and O/O) [21,22]. The risk of infection was investigated for (i) bloodstream infection (BSI), (ii) pneumonia and (iii) BSI or pneumonia or both. Analysis was performed using multiple logistic regression with the

potential risk-factors for nosocomial infection that had been evaluated in the previous surveillance study in the NICU of the VU University Medical Centre [17]. These factors were birth-weight category (<1000 g, 1000–1499 g, 1500–2499 g, ≥2500 g), gender, place of birth (VU University Medical Centre or elsewhere), type of birth (Caesarian section or vaginal), presence of meconium-stained amniotic fluid, premature rupture of membranes (>24 h before delivery) and multiple birth. Statistical analysis was performed using SPSS v.12.0 (SPSS Inc., Chicago, IL, USA).

Genotype distribution, according to Garred *et al*. [21] and Neth *et al*. [22], was compared for pre-term neonates and term neonates. Further analysis investigated whether significant differences existed between early (gestational age <32 weeks) and late (gestational age 32–36 weeks) pre-term neonates.

RESULTS

Parents of 512 neonates were asked to give informed consent for participation in this study. Ultimately, 204 Guthriecards were investigated, with 188 cases yielding sufficient DNA after pre-PCR for further analysis. In general, the results for estimation of SNPs were good; however, no result was obtained in 11 of the $6 \times 188 = 1128$ SNP estimations, although the MBL genotype could still be deduced in most cases. In one of the 188 DNA specimens, the combinations of SNPs could not be translated into known haplotypes according to Garred *et al*. [21] and Neth *et al*. [22]. This specimen had the combination L/H–Y/X–P/P–A/A–no result–A/A. There was another specimen for which each single SNP was estimated, but which could not be allocated to one of the seven well-known haplotypes. Thus, 186 MBL genotypes were investigated in relation to infection risk; 35 (19%) of 186 neonates had two low-level MBL haplotypes (LXPA, LYPB, HYPD or LYQC), and 69 (37%) had one low-level haplotype.

Results of bacterial cultures were retrieved from the previous study [17] and are shown in Table 1. Coagulase-negative staphylococci (CoNS) were the most frequent cause of BSI. There was no predominant causative microorganism for pneumonia. In total, 41 neonates had BSI, and 30 had pneumonia; eight neonates had BSI as well as pneumonia. The MBL genotypes for infected and non-infected neonates are shown in Table 2. In a univariate analysis, as well as in a multiple analysis with other baseline risk-factors for nosocomial infection, no relationship was found between the MBL genotype of the neonates and the risk of infection. Birth-weight was the only significant risk-factor for nosocomial infection (Table 3).

Table 1. Causative organisms of infection

	Causative organism	*n*
Bloodstream infection	Coagulase-negative staphylococci	25
	Bacillus cereus	3
	Staphylococcus aureus	3
	Viridans group streptococci	1
	Enterobacter cloacae	1
	No organism isolated	8
Total		41
Pneumonia	*Ureaplasma urealyticum*	4
	Bacillus spp.	4
	Mixed Gram-positive/negative flora	4
	Klebsiella spp.	3
	Staphylococcus aureus	2
	Haemophilus influenzae	1
	Enterobacter cloacae	1
	Escherichia coli	1
	Candida albicans	1
	No organism isolated/no significant pathogens	9
Total		30

Table 2. Mannose-binding lectin (MBL) genotypes in relation to the occurrence of infection

	Infection					
	Pneumonia		BSI		BSI or pneumonia or both	
MBL genotype	Yes	No	Yes	No	Yes	No
Low MBL genotypes (%)						
O/O	4 (13.3)	10 (6.4)	3 (7.3)	1 (7.6)	6 (9.5)	8 (6.5)
XA/O	0	15 (9.6)	4 (9.8)	11 (7.6)	4 (6.4)	1 (8.9)
XA/XA	0	9 (5.8)	1 (2.4)	8 (5.5)	2 (3.2)	7 (5.7)
Total	4 (13.3)	34 (21.8)	8 (19.5)	30 (20.7)	12 (19.0)	26 (21.1)
Medium MBL genotypes (%)						
YA/O	6 (20.0)	26 (16.7)	5 (12.2)	27 (18.6)	10 (12.2)	22 (17.9)
YA/XA	10 (33.3)	33 (21.2)	10 (24.4)	33 (22.8)	16 (25.4)	27 (22.0)
Total	16 (53.3)	59 (37.8)	15 (36.6)	60 (41.4)	26 (41.3)	49 (39.8)
High MBL genotype (%)						
YA/YA	10 (33.3)	63 (40.4)	18 (43.9)	55 (37.9)	25 (39.7)	48 (39.0)
Total (n = 186)	30 (100)	156 (100)	41 (100)	145 (100)	63 (100)	123 (100)

BSI, bloodstream infection.

No significant differences were revealed between individual low and medium MBL genotypes among term and pre-term neonates. However, the wild-type genotype was significantly more prevalent among term neonates; thus mutant genotypes, overall, occurred significantly more frequently among the pre-term neonates. No differences were detected between early and late pre-term neonates.

DISCUSSION

MBL is present in the serum of pre-term neonates and in amniotic fluid, and the amount of MBL

Table 3. Multivariate analysis of the mannose-binding lectin (MBL) genotype in relation to the risk of infection[a]

		BSI OR (95% CI)		Pneumonia OR (95% CI)		BSI or pneumonia or both OR (95% CI)	
	n	*n* (%)	OR (95% CI)	*n* (%)	OR (95% CI)	*n* (%)	OR (95% CI)
Promotor exon genotype [21]							
YA/YA	72	18 (25)	Reference group	10 (14)	Reference group	25 (35)	Reference group
YA/XA	44	10 (23)	0.59 (0.22–1.61)	10 (23)	1.54 (0.56–4.22)	16 (36)	0.71 (0.28–1.77)
XA/XA	9	1 (11)	0.45 (0.04–4.93)	0	NA	2 (22)	0.24 (0.02–2.59)
YA/O	32	5 (16)	0.46 (0.14–1.53)	6 (19)	1.42 (0.45–4.51)	10 (31)	0.72 (0.26–2.02)
XA/O	15	4 (27)	1.64 (0.39–6.94)	0	NA	4 (27)	0.87 (0.21–3.58)
O/O	14	3 (21)	0.53 (0.11–2.46)	4 (29)	2.16 (0.53–8.81)	6 (43)	1.01 (0.26–3.95)
Total	186	41 (22)		30 (16)		63 (34)	
Birth-weight category			3.65 (2.25–5.92)		2.16 (1.36–3.45)		3.73 (2.40–5.75)
Exon genotype [22]							
A/A	125	29 (23)	Reference group	20 (16)	Reference group	43 (34)	Reference group
A/O	47	9 (19)	0.89 (0.35–2.23)	6 (13)	0.85 (0.31–2.34)	14 (30)	0.94 (0.41–2.14)
O/O	14	3 (21)	0.68 (0.16–2.97)	4 (29)	1.92 (0.51–7.23)	6 (43)	1.24 (0.34–4.57)
Total	186	41 (22)		30		63 (34)	
Birth-weight category			3.42 (2.15–5.46)		2.26 (1.43–3.57)		3.66 (2.39–5.65)

BSI, bloodstream infection; NA, not applicable.
[a]For estimation of the infection risk, the wild-type genotype was used as the reference group.

increases with the duration of gestation [23,24]. MBL is also detectable in cord blood [25], but it is thought that there is no passive transfer of maternal MBL to the foetus [26]. MBL levels increase during the first 3 months of life in response to environmental changes [27,28], and recurrent infection has been described in MBL-deficient infants [29].

An association was found in the present study between the prevalence of low-MBL-producing genotypes and pre-term birth, but no relationship was revealed between low-MBL-producing genotypes and the risk of nosomial infection. It is known that pre-term neonates have lower MBL concentrations than term neonates [30,31], and that the adaptive immune system in this patient category functions sub-optimally, perhaps because of lowered production of granulocyte colony-stimulating factor, leading to a low number of neutrophils [32]. The phagocytic function of those neutrophils is also impaired [11,12,15], as is opsonisation by IgG [33] and chemotaxis [13].

In contrast to the present findings in pre-term neonates, MBL deficiency has been described as a risk-factor for increased susceptibility to infection in adults and older children, especially when combined with another immunocompromising factor, e.g., chemotherapy or haematological malignancy [2,3,6,22,34–36]. MBL-variant alleles have also been found to be a risk-factor for acute respiratory illness in children aged 6–17 months, which is an age at which the protecting maternally-acquired IgG antibodies have disappeared and the adaptive immune system is still immature [3]. Therefore, the lack of a correlation in the present patient population was unexpected. However, Ahrens *et al.* [37] also failed to find a relationship between mutations in the *MBL* gene and sepsis in premature neonates, but reported a clear correlation with gestational age. Unfortunately, no information concerning causative microorganisms for infection related to MBL genotype was provided.

There are several hypotheses to explain the absence of any relationship between the risk of infection and the MBL genotype in pre-term infants requiring intensive care treatment, despite their premature adaptive immune systems. First, there was a high prevalence of BSIs caused by CoNS in this patient group. It is known that MBL binds poorly to CoNS [38], and it is therefore likely that complement activation through MBL binding is not effective in infections caused by these microorganisms, so that MBL is not protective. Because the number of patients with infections caused by microorganisms other than CoNS was low in the present study, a possible protective effect of MBL in such infections might not be detectable. The meaning of a single blood culture positive for CoNS is debatable, but because of the small circulating volume in pre-term neonates, it is not common practice to obtain additional blood cultures for sepsis investigations. CDC-approved definitions were used for microbiologically confirmed BSI, which include the isolation of a bacterium belonging to the normal skin flora in the presence of a central venous catheter, which was always the case in the present study [18]. Second, the MBL genotype may not be a good predictor of MBL function in individual patients

because of significant individual variability in the correlation between genotype and MBL function [6]. Even normal MBL levels can be associated with a non-functional MBL system because of, e.g., a deficiency in MBL-associated serine protease 2 [39]. Furthermore, individuals with an identical genotype may differ in MBL serum levels by up to ten-fold [40].

Functional MBL pathway assays have now been developed, and these have led to a better understanding of the relationship between genotype and levels of functional MBL. It appears that, especially with heterozygosity, the amount of MBL in the circulation is less dependent on the genotype than was predicted previously [41,42]. Only high molecular size MBL activates the complement system efficiently. The MBL function in heterozygous individuals may therefore be highly dependent on the promoter function of the wild-type allele [42].

The present study inferred abnormalities in the MBL levels, based on the different haplotypes that were determined by SNP analysis. The nested PCR approach used with DNA recovered from Guthriecards was very efficient, and the technique enabled MBL alleles to be determined for neonates after they had been discharged from hospital, without a need to obtain new blood specimens. One neonate had a combination of SNPs that did not belong to the six well-known haplotypes investigated in the present study, but additional MBL haplotypes have been described recently [43]. Overall, it was concluded that the six MBL haplotypes investigated did not correlate with the risk of infection in pre-term neonates in an NICU in which the majority of infections were caused by CoNS.

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